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Development of a base editor for convenient and multiplex genome editing in cyanobacteria

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Cyanobacteria are important primary producers, contributing to 25% of the global carbon fixation through photosynthesis. They serve as model organisms to study the photosynthesis, and are important cell factories for synthetic biology. To enable efficient genetic dissection and metabolic engineering in cyanobacteria, effective and accurate genetic manipulation tools are required. However, genetic manipulation in cyanobacteria by the conventional homologous recombination-based method and the recently developed CRISPR-Cas gene editing system require complicated cloning steps, especially during multi-site editing and single base mutation. This restricts the extensive research on cyanobacteria and reduces its application potential. In this study, a highly efficient and convenient cytosine base editing system was developed which allows rapid and precise C → T point mutation and gene inactivation in the genomes of *Synechocystis* and *Anabaena*. This base editing system also enables efficient multiplex editing and can be easily cured after editing by sucrose counter-selection. This work will expand the knowledge base regarding the engineering of cyanobacteria. The findings of this study will encourage the biotechnological applications of cyanobacteria.

Cyanobacteria are the only oxygenic photosynthetic prokaryotes that can convert CO_2 into organic compounds using light as the sole energy source[1]. Some cyanobacterial strains serve as important model organisms to study the physiological and ecological phenomena, such as photosynthesis, biological nitrogen fixation, and prokaryotic cell differentiation[2–4]. In addition, cyanobacteria have attracted particular attention as promising cell factories for sustainable generation of a large variety of valuable bioproducts, such as biofuels[5,6], commercial terpenoids[7,8], bioplastics[9], bioactive compounds[10,11], sugars[12,13], and pigments[14]. To enable efficient genetic dissection and rational metabolic engineering in cyanobacterial strains, reliable genetic manipulation tools are required to allow precise, marker-less, rapid, and multiplex editing of genome. However, the conventional allelic-exchange-based genome editing method available for cyanobacteria is time-consuming, and leaves antibiotic markers at the editing sites[15], which can be eliminated by subsequent selection processes[16]. (Fig. 1a). Thus, lack of reliable genetic manipulation tools has become a major hurdle in understanding the basic processes and metabolic networks of cyanobacteria. This restricts the potential of cyanobacteria in environmental and biotechnological research and applications.

The recent developments in the CRISPR-Cas system provide a simple and reliable platform for precise and efficient modification of DNA sequences in a wide range of organisms, including mammals[17], plants[18,19], and bacteria[20–22]. CRISPR-Cas9 is the most commonly used CRISPR system for genome editing[23,24]. In this system, the Cas9 nuclease forms a complex with crRNA and tracrRNA, or with an artificial single guide RNA (sgRNA). Then, the complex is guided to the specific complementary target DNA site which must be next to a protospacer adjacent motif (PAM, e.g., 5'-NGG-3' for *Streptococcus pyogenes* Cas9). Thus, a blunt double-strand break (DSB) is formed in the genome[23,25] (Fig. 1b). The common method to repair the lethal DSBs in prokaryotes is homology-directed repair (HDR). In this method, a repair template is provided exogenously, and sequence-specific deletions and insertions are achieved during the repair process. Cpf1 (also named Cas12a) is another CRISPR-Cas system, which recognizes a 5' T-rich PAM (e.g., 5'-TTN-3' for *Francisella novicida* Cpf1), and generates staggered double-strand DNA break without the assistance of tracrRNA[26] (Fig. 1c).

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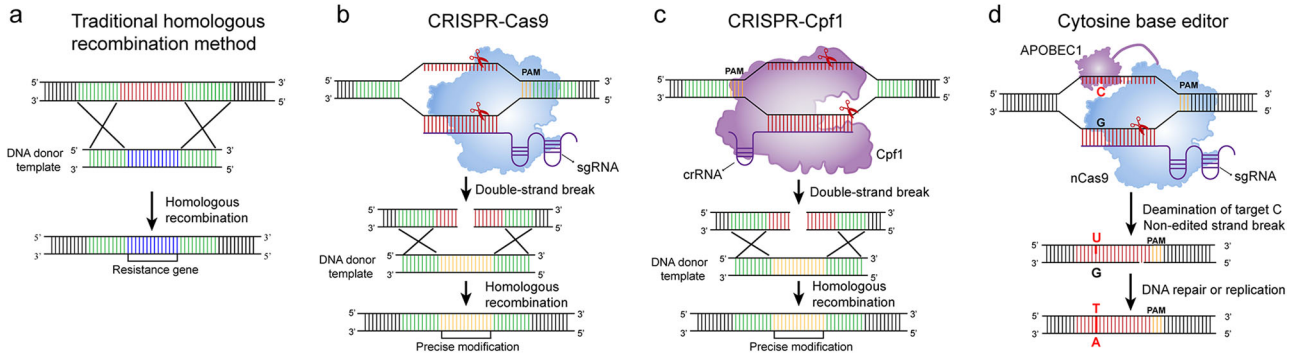

Fig. 1 | Comparison of different genome editing methods in cyanobacteria. a Traditional homologous recombination method; **b** CRISPR-Cas9 method; **c** CRISPR-Cpf1 method; **d** Cytosine base editor.

Both Cas9 and Cpf1 have been employed for the genome editing in cyanobacteria[27]. Cas9-mediated genome editing system was first applied in cyanobacteria, such as *Synechococcus* sp. PCC 7942[28], *Synechococcus* sp. PCC 2973[29], and *Synechocystis* sp. PCC 6803[30]. Since high dosage of Cas9 is cytotoxic to cyanobacteria and causes low transformation efficiencies[31], inducible promoter was used to strictly control the Cas9 expression, which enabled reliable transformation of the CRISPR-Cas9 vector in cyanobacteria[30,32]. In contrast, Cpf1 is much less toxic to cyanobacteria, and has been successfully used for different cyanobacterial species, including *Synechococcus*, *Synechocystis*, and *Anabaena*[33–35]. Although Cas9 and Cpf1 enable efficient and scarless genome editing in cyanobacteria, they still rely on the HDR mechanism which can reduce the transformation efficiency and requires multiple cloning steps to assemble the homologous recombination template, especially during multi-site editing.

Recently, the development of "base editors" allows direct base mutagenesis at specific genomic sites, providing a distinctive strategy for genome editing[36]. Until now, several kinds of base editors have been developed[37–41]. Among them, the cytosine base editor is the most popular one, which has been applied in a number of organisms[37,42–44]. A cytosine base editor is engineered by fusing a cytidine deaminase with a dead Cas9 (Cas9D10AH840A) or a Cas9 nickase (Cas9D10A). Guided by the Cas9/sgRNA complex, the cytidine deaminase is directed to the specific target site, and a C → T conversion (or G → A in the complementary strand) is achieved (Fig. 1d). By mediating the conversion of CAA, CAG, CGA, or TGG to TAA, TAG, or TGA, cytosine base editor can generate a premature stop codon at the target site, thus inactivating the target gene. This genome editing tool does not create DSBs, and thus it enables efficient genetic manipulation without sacrificing the transformation efficiency. In addition, it does not require the donor repair template for the homologous recombination repair. Therefore, simply by customizing approximately 20 nucleotides (nt) of the sgRNA, the cytosine base editor can edit specific cytosine bases of interest in the genome, which makes it an easily programmable tool for precise genome editing and gene inactivation. Compared with conventional homologous recombination method and Cas9/Cpf1-based genome editing tools, the base editor system has some advantages, such as a simple cloning process, rapid editing speed, and multiplex editing capability (Table 1).

In this study, a highly efficient and convenient cytosine base editing system (pCyCBE) was developed in cyanobacteria. This tool allowed rapid and precise point mutation and gene inactivation in the genome of cyanobacteria. It also enabled efficient multiplexed editing and could be easily cured by sucrose counter-selection. The development of the pCyCBE system will pave the way for future physiological studies and metabolic engineering in cyanobacteria. The findings of this study will provide critical insights into base-editing system development in other microorganisms.

Results

Construction of the pCyCBE plasmid for base editing in cyanobacteria

To harness the cytosine base editor for genome editing in cyanobacteria, a base editor plasmid was constructed by using pBECKP-Km[45] and pCpf1b-Sp[34] as the PCR template (Fig. 2). The expression cassette of the base editor was amplified from the pBECKP-Km plasmid, a cytidine deaminase-mediated base-editing plasmid in *Klebsiella Pneumoniae*. The pCpf1b-Sp plasmid is a Cpf1-based plasmid for gene editing in cyanobacteria, and its plasmid backbone was amplified for the replication in cyanobacteria. The two DNA fragments were assembled together via Gibson assembly, resulting in the plasmid pCyCBE. The constructed pCyCBE plasmid contains several components: the broad host range replicon RSF1010 that enables the plasmid to replicate in cyanobacteria; the kanamycin-resistant marker for selection; the cytidine deaminase APOBEC1 linked to the N-terminus of the nickase Cas9D10A via an XTEN linker; the sgRNA, driven by the promoter J23119, a strong constitutive promoter in prokaryotes; and the counter-selection gene *sacB* for the plasmid curing after editing. In addition, two *Bsa*I sites were designed in the plasmid for convenient and seamless cloning of spacers by Golden Gate assembly (Fig. 2).

Efficient C → T conversion in *Synechocystis* sp. PCC 6803 mediated by the pCyCBE plasmid

Base editing capacity of the pCyCBE system was analyzed in the model cyanobacterium *Synechocystis* sp. PCC 6803. We selected two non-essential genes, *hlyD* (*sll1181*) and *desB* (*sll1441*), to attempt base editing. Previous studies have shown that HlyD is an important component of the HlyBD-TolC efflux system and is involved in the adaptation of low-iron (Fe) conditions in *Synechocystis* sp. PCC 6803[46], while DesB is a fatty acid desaturase and is essentially required for low-temperature adaptation[47]. Two spacers were designed to target the *hlyD* and *desB* genes, and then were assembled into the pCyCBE plasmid, respectively. After transforming the editing plasmids into *Synechocystis* sp. PCC 6803 by conjugation, individual clones grown on the BG11 agar plate were picked and the editing efficiency was calculated by analysis of the Sanger sequencing chromatogram using the EditR software[48]. As shown in Fig. 3a, the C at position 8 of the *hlyD* spacer and the C at position 6 of the *desB* spacer were successfully mutated to T with editing efficiencies of 93.7% and 92.4%, respectively. This indicated that the pCyCBE system could efficiently mediate the base editing in *Synechocystis* sp. PCC 6803. To accurately measure the editing efficiency of the pCyCBE base editor, we further amplified the target sites of the mutant strains, and sent the PCR products for deep sequencing. The results showed that the editing efficiencies of the target sites were approximately 99% (Fig. 3b), indicating that almost all of the chromosome copies were mutated.

The C → T conversion in *hlyD* gene generates a premature stop codon (Q58 to stop codon), resulting in the inactivation of the *hlyD* gene. Since HlyD is involved in the formation of type IV pili and indirectly influences Fe

Table 1 | Comparison of different genome editing systems in cyanobacteria

	Conventional	Cas9	Cpf1 (Cas12a)	Base editor
Editing method	Homologous recombination	Cas9-induced DSB and homologous recombination	Cpf1-induced DSB and homologous recombination	Deaminase-mediated base conversion, no DSB
Typical length of procedure	From 3 weeks to several months until segregation	From 9 days to a few weeks until segregation	From 9 days to a few weeks until segregation	From 9 days to a few weeks until segregation
Cloning steps	Multiple	Multiple	Multiple	One step
Editing effect	Deletion, insertion, and mutation	Deletion, insertion, and mutation	Deletion, insertion, and mutation	Base mutation
Completeness of gene knockout	100%	100%	100%	<100%
Homology arm	Needed	Needed	Needed	Not needed
Multiplex editing	No	Yes	Yes	Yes
Toxic to cyanobacteria	Low	Relatively high	Low	Low

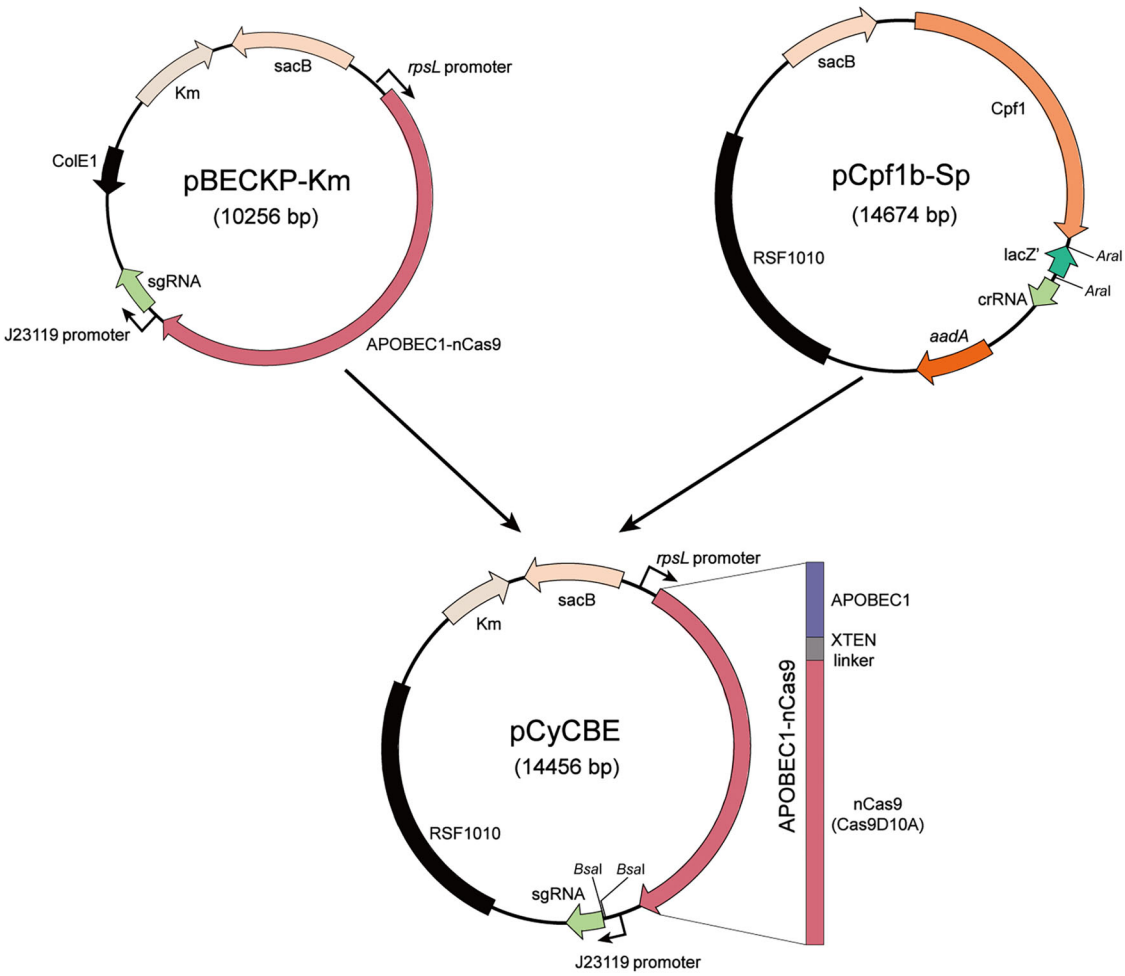

Fig. 2 | Construction and characteristics of pCyCBE plasmid. The pCyCBE plasmid is constructed by substituting ColE1 origin in pBECKP-Km with the RSF1010 origin of pCpf1b-Sp.

acquisition, the *hlyD* mutant strain is more sensitive to Fe deficiency and loses the ability of phototactic movement[46]. To verify the phenotype of the *hlyD* mutant constructed by the base editor, the growth curve assay was conducted in Fe depletion conditions. As shown in Fig. 3c, the *hlyD* mutant showed similar growth rate as the unedited strains under standard Fe conditions. However, the *hlyD* mutant strain grew much slower than the unedited strains under Fe-deficient conditions. In addition, the *hlyD* mutant lost the ability of phototactic movement (Fig. 3d). These results are consistent with the finding reported by the previous studies based on the traditional homologous recombination method of gene disruption[46], suggesting that the *hlyD* gene was indeed inactivated by the pCyCBE base editing system.

To elucidate the kinetics of base editing, we measured the editing efficiencies of *hlyD* and *desB* genes after transformation at different time points (the 7th day, 12th day, and 20th day). As shown in Supplementary Fig. 1, the editing efficiency continued to increase over time. At the 20th day, the editing efficiencies of *hlyD* and *desB* genes reached over 90%, indicating that most of the chromosome copies were edited.

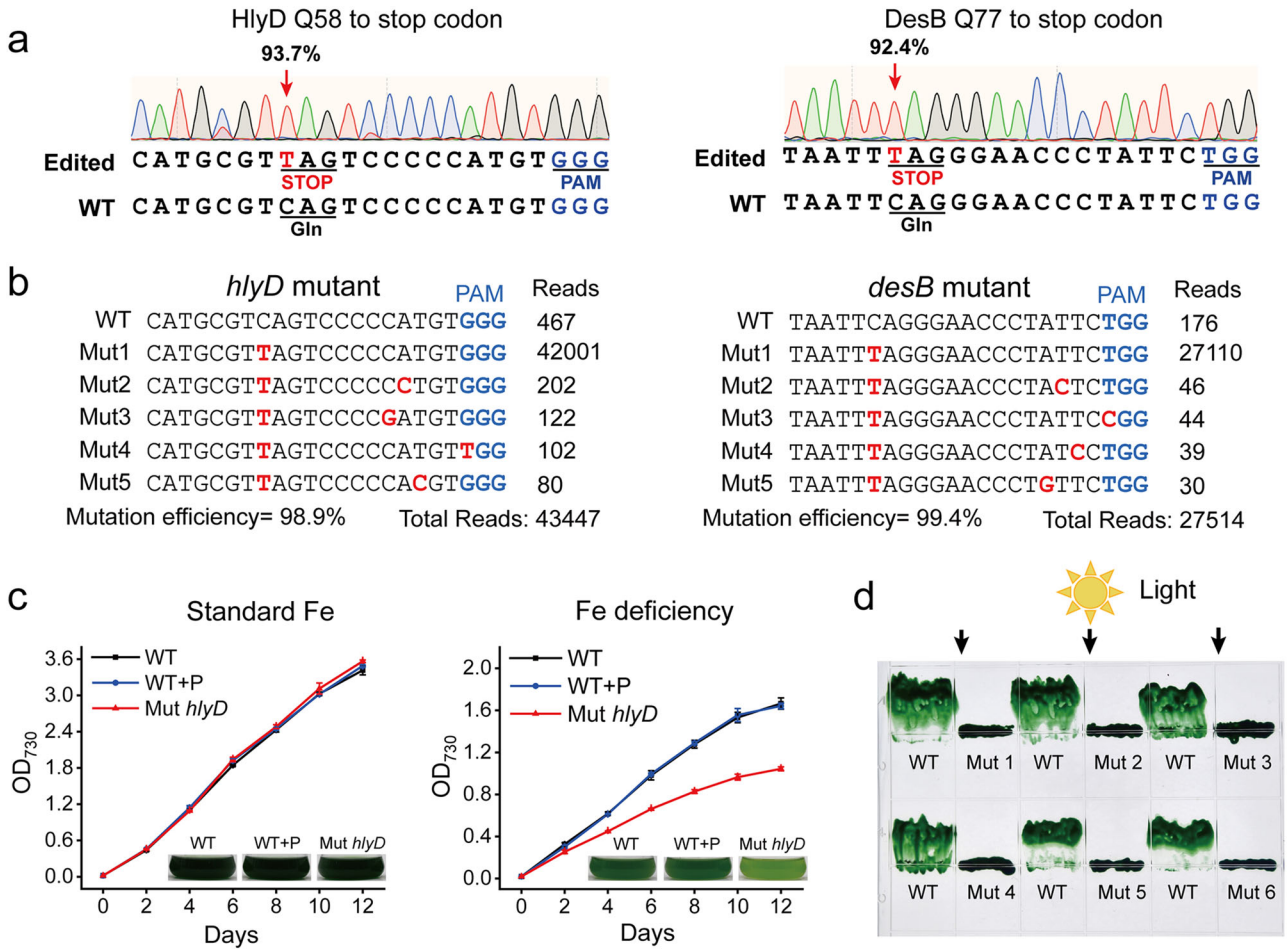

Fig. 3 | The pCyCBE base editing plasmid mediating efficient C → T conversion in *Synechocystis* sp. PCC 6803. a HlyD Q58 and DesB Q77 in the genome of *Synechocystis* sp. PCC 6803 are successfully mutated to stop codon; PAM sites are highlighted in blue color, while mutation sites are shown in red color. Each base editing experiment is repeated three times, and the representative sequencing chromatogram for each mutation is shown. **b** Deep sequencing results of the *hlyD* and *desB* mutant strains. The top 6 most frequent PCR products are displayed. **c** Growth curves of the wild-type (WT) strain, wild-type strain carrying the pCyCBE plasmid (WT + P), and the *hlyD* mutant (Mut *hlyD*) strain grown under standard Fe and Fe-deficient conditions. Photographs of the cultures on the 6th day are presented at the bottom of the growth curve. The growth curve experiments are performed in triplicate, and the data are represented as mean ± SD. **d** Phototactic movements of the WT strain and Mut *hlyD* strain grown for 7 days under lateral illumination.

To systematically investigated the versatility of the pCyCBE base editor system in *Synechocystis* sp. PCC 6803, 18 different spacers were designed, targeting different genomic sites (Supplementary Table 1). All of the cyanobacterial colonies grown on the BG11 plate were collected for PCR and the integral editing efficiencies were examined using Sanger sequencing. The results showed that 17 spacers were successful edited (Fig. 4a). Furthermore, we calculated the editing efficiency of all the Cs at the 20 bp spacer by analysis of the Sanger sequencing chromatogram using the EditR software[48]. As shown in Fig. 4b, editing events occurred across the 20 positions on the spacer. However, these events were particularly concentrated at the 4-9 positions, which indicated that these positions in the spacer were most likely to be edited (referred as "editable window"). All the Cs in the "editable window" were further selected, and the influence of adjacent bases on the editing efficiency was analyzed. The editing efficiencies were found to be in the following order: TC > CC ≈ AC > GC (Fig. 4c), which was consistent with the previous studies[37,44]. These results suggested that TC is a better mutation site at the "editable window" while designing the suitable spacers for genome editing.

Efficient multiplex editing in cyanobacteria by the base editor

Considering the high editing efficiencies of the pCyCBE system in *Synechocystis* sp. PCC 6803, capability of multiplex base editing was further examined using tandem gRNA cassettes. Firstly, two sgRNAs, targeting *hlyD* and *desA* (*slr1350*) genes, were assembled into the pCyCBE plasmid simultaneously (Fig. 5a). The results of Sanger sequencing showed that these two genes were simultaneously mutated with high editing efficiency (Fig. 5a). Another two-site editing plasmid targeting *hlyD* and *tolC* (*slr1270*) genes also showed successful editing at both target sites (Supplementary Fig. 2a). Furthermore, three sgRNAs were assembled into the pCyCBE plasmid, resulting in pCyCBE-3xsgRNA-1, targeting *desA*, *desB*, and *desD* (*sll0262*) genes, and pCyCBE-3xsgRNA-2, targeting *desA*, *desB*, and *desC* (*sll0541*) genes. The Sanger sequencing results confirmed successful mutations at the targeted sites (Fig. 5b, Supplementary Fig. 2b). These results indicate that the pCyCBE system is capable of efficient base editing at multiple sites simultaneously.

The editing plasmid could be easily cured by sucrose counter-selection

After editing, the plasmid should be cured to construct a marker-less mutant strain for phenotypic analysis without any genetic interference from unrelated genetic background, and for the subsequent editing of other genomic sites. The pCyCBE plasmid contains *sacB* gene, which acts as a lethal gene in the presence of sucrose. Thus, this plasmid can be easily cured by using agar plates containing sucrose. To cure the pCyCBE plasmid after editing, the

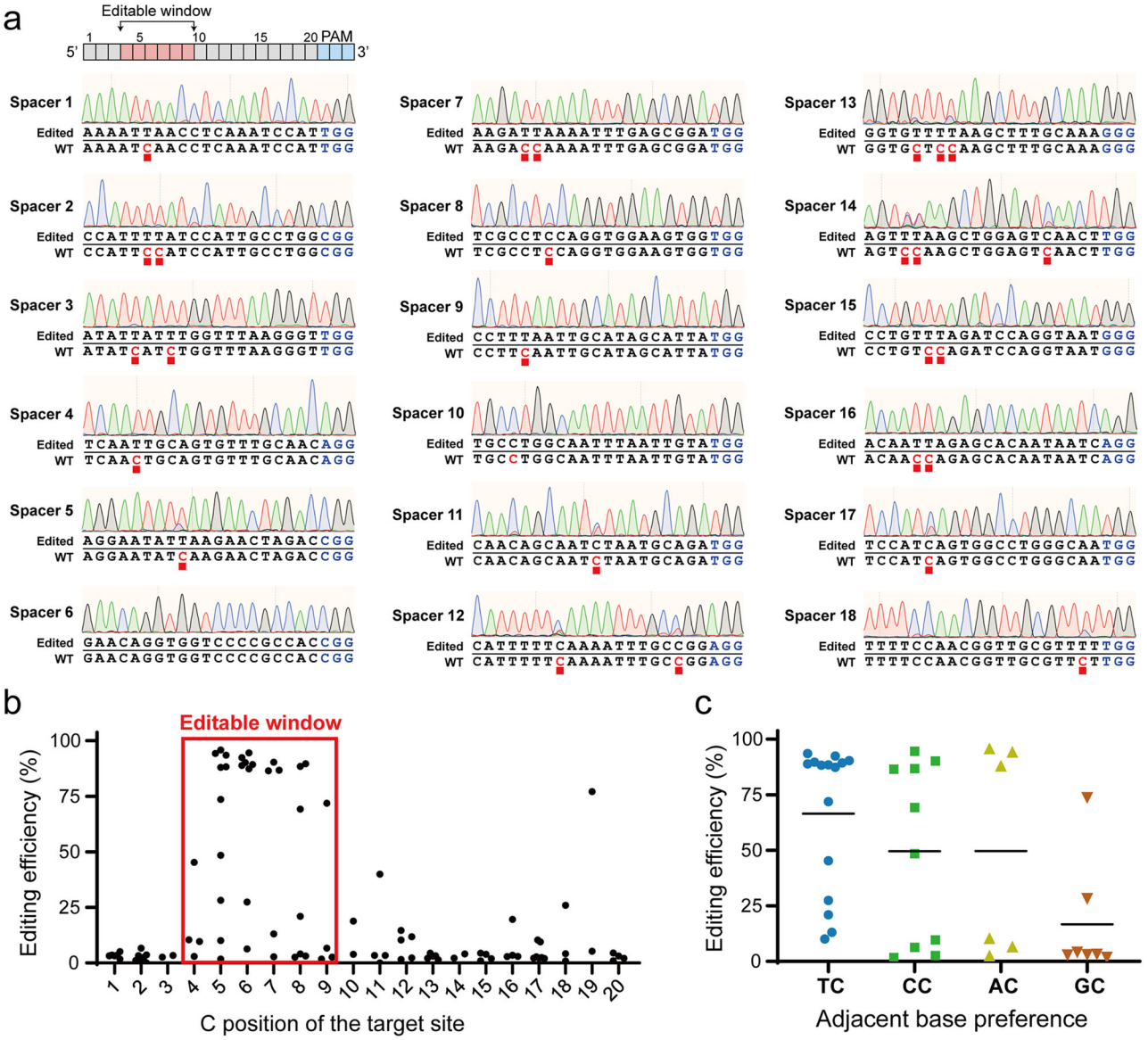

Fig. 4 | Systematic analysis of the pCyCBE base editor in *Synechocystis* sp. PCC 6803. a Sequencing chromatograms of the selected 18 target loci in the pCyCBE system. The edited C(s) in the spacer are colored in red and indicated by red box. **b** Summary of the base-editing frequency at each cytidine site at the tested sites, with "editable window" being located between positions 4 and 9 in the spacer region, and labeled with red box. **c** Analysis of the adjacent base preference of the pCyCBE system in *Synechocystis* sp. PCC 6803.

hlyD mutant strain was cultured in fresh BG11 medium for 4 generations. Then the culture was diluted 10^4 and 10^5 folds with fresh BG11 medium. Subsequently, 50 μL of diluted culture was poured onto the BG11 agar plates in the presence of 5% w/v sucrose. At the same time, diluted culture was also plated on the BG11 agar medium without sucrose. The number of colonies grew on the sucrose-free plate was much more than that on the plate containing sucrose (Fig. 6a). This indicated the elimination of many plasmid-containing colonies by sucrose. Furthermore, 6 colonies were randomly picked from the plates with or without sucrose, and PCR was performed to verify the loss of plasmid using pCyCBE plasmid-specific primers. As shown in Fig. 6b, all colonies picked from the sucrose-free plate contained the editing plasmid, while the colonies from the sucrose plate lost the editing plasmid. Subsequently, the colonies picked from the sucrose plate were streaked on the BG11 agar plate with or without kanamycin, respectively. All the colonies grew well on the antibiotics-free plate, whereas the sucrose selected strains could not grow in presence of kanamycin (Fig. 6c). This confirmed that the editing plasmid was successfully cured in presence of sucrose. Overall, the pCyCBE plasmid could be easily cured by the counter-selection of sucrose after genome editing.

Assessment of the gene editing stability and off-target effect of the pCyCBE system

To assess the gene editing stability of the pCyCBE base editor, we cultured three mutant strains (*hlyD*, *desB*, and *desA*) after plasmid curing in standard BG11 medium for 20 generations, respectively. The Sanger sequencing results showed that all three genes were completely mutated and no unedited event were observed after 20 generations of cultivation (Supplementary Fig. 3a–c), indicating that the mutant strains were stable. In addition, the growth curve assay was performed using the *hlyD* mutant strain cultured for 20 generations. The results showed that the 20th-generation *hlyD* mutant showed similar growth rate as the wild-type strain under standard Fe conditions, but grew much slower than the wild-type strain under Fe-deficient conditions (Supplementary Fig. 3d), which is in consistent with the phenotype of the 1st-generation *hlyD* mutant. Taken together, these

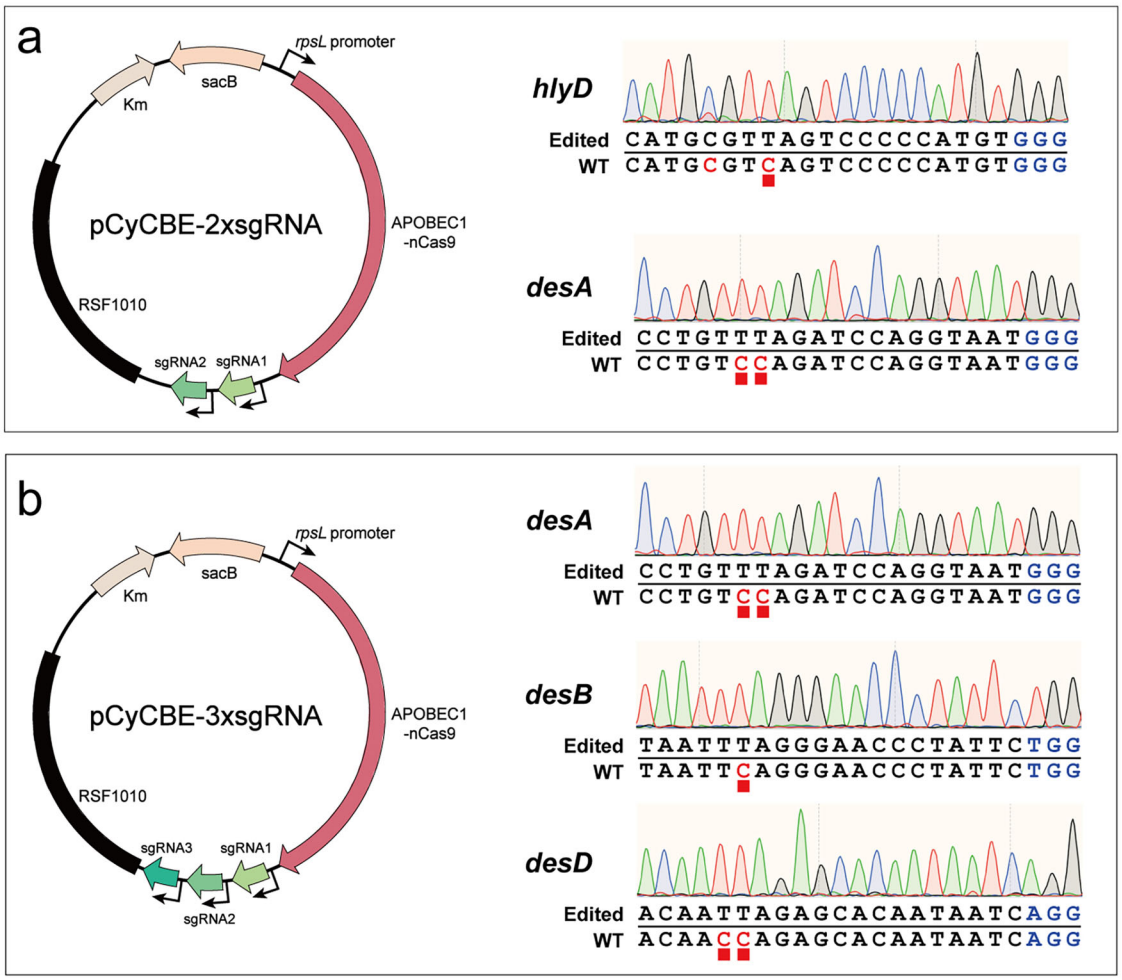

Fig. 5 | Efficient multiplex editing in cyanobacteria, mediated by pCyCBE plasmid. a The pCyCBE plasmid enables simultaneous base editing at two sites. A Map of pCyCBE-2xsgRNA plasmid is presented in the left, and the representative sequencing chromatograms of two mutation sites are presented in the right. **b** The pCyCBE plasmid enables simultaneous base editing at three sites. Map of pCyCBE-3xsgRNA plasmid is presented in the left, and the representative sequencing chromatograms of three mutation sites are presented in the right. The mutated Cs are shown in red color and labeled with red squares.

results demonstrated that the genomic mutations generated by the pCyCBE base editor is stable, and would not be rapidly lost during the cultivation process.

To assess the off-target effect of the pCyCBE system, we searched the similar genomic sites of four spacers (*hlyD*, *desB*, *desA*, and *desC*) across the entire genome of *Synechocystis* sp. PCC 6803 using the CasOFFinder software[49]. We picked the top five similar sites for each gene (Supplementary Table 2), and amplified these genomic loci of the mutant strains for Sanger sequencing. The sequencing results showed that none of the similar genomic sites were mutated (Supplementary Fig. 4a–d), indicating that the pCyCBE system has good fidelity. Nowadays, the off-target effects of base editors in plant and mammalian cells have been reported[50–52]. Apart from the sgRNA-dependent off-target DNA editing, the cytosine base editor also generates undesired mutations by sgRNA-independent off-target effect, which is caused by random deamination without the participation of sgRNA[50,52]. Therefore, to comprehensively evaluate the off-target effects of base editor in cyanobacteria, whole genome sequencing might be an effective method.

Application of the pCyCBE system in other cyanobacterial strains

RFS1010 origin is a replicon with broad host range, and it can replicate in different cyanobacterial species. This indicates that the pCyCBE plasmid can also mediate base editing in other cyanobacterial species, apart from *Synechocystis* sp. PCC 6803. To verify this hypothesis, the pCyCBE system was applied in another model cyanobacterium *Anabaena* sp. PCC 7120. It is a filamentous strain, which is able to form heterocysts to fix atmospheric nitrogen under the conditions of limited nitrogen in the growth media. Six target sites in the non-essential genes were designed, and the constructed plasmids were transferred into *Anabaena* sp. PCC 7120 by conjugation. The results of Sanger sequencing showed successful mutations at all selected sites with high editing efficiencies (Fig. 7a, Supplementary Fig. 5). HetF is a membrane protein which is essential to heterocyst formation in *Anabaena* sp. PCC 7120 and the *hetF* deletion strain displayed an aberrant cell morphology characterized by enlarged and elongated cells[53]. The C → T conversion in the *hetF* (*alr3546*) gene mutated the Q58 to stop codon, generating *hetF* mutant strain. Consistent with the previous study[53], the *hetF* mutant strain displayed an aberrant cell morphology, with enlarged and elongated cells in the BG11 medium (Fig. 7b). To seriously analyze the differences of the wild-type and *hetF* mutant strains in the cell dimensions, we randomly selected 100 cells from each strain, and measured their cell lengths under microscope. The results showed that the lengths of the *hetF* mutant strain were significantly longer than that of unedited strains ($P < 0.001$) (Fig. 7c). These results confirmed that the *hetF* gene in *Anabaena* sp. PCC 7120 was successfully disrupted by the pCyCBE base editor.

To estimate the number of genes that could be inactivated by the pCyCBE system in cyanobacteria, targetable codons in the genome of *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 were determined

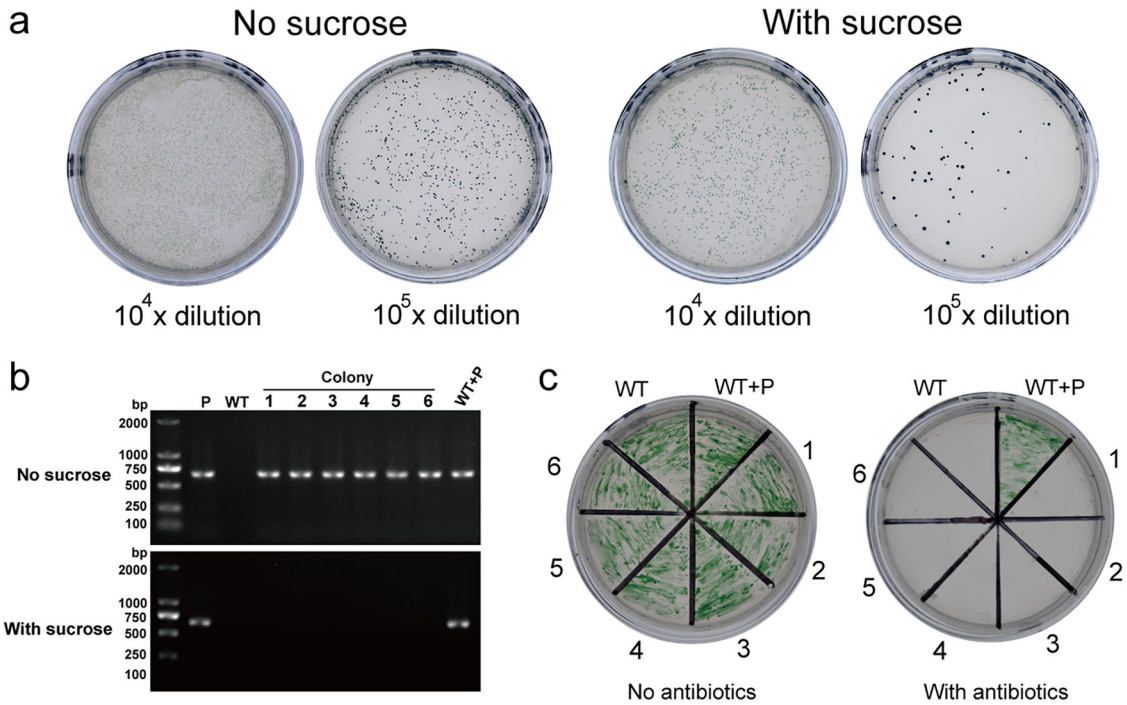

Fig. 6 | Curing of the pCyCBE plasmid in cyanobacteria after editing. **a** Photographs of the edited strains grown on the BG11 agar plate in the absence and presence of sucrose. **b** PCR results of the six colonies randomly chosen from the BG11 plates with or without sucrose. Here, P is the pCyCBE plasmid; WT is the wild-type strain; WT + P is the wild-type strain containing pCyCBE plasmid. **c** All six colonies chosen from the sucrose plate are sensitive to the antibiotic (kanamycin).

using the CRISPR-CBEI software[54]. As shown in Fig. 7d, the premature stop codon could be introduced into over 90% genes of both two strains by the pCyCBE base editor. Considering that the relative position of the premature stop codon in an open reading frame (ORF) can significantly affect the effectiveness of gene inactivation, the locations of the introduced premature stop codon in the gene were further analyzed. The results showed that at least one premature stop codon could be introduced in around 93.75%, 87.87%, and 71.58% genes of *Synechocystis* sp. PCC 6803, within the top 75%, 50%, and 25% of the ORF body, respectively. Whereas in *Anabaena* sp. PCC 7120, a premature stop codon could be introduced in 87.76%, 80.07%, and 61.12% genes, within the top 75%, 50%, and 25% of the ORF body, respectively (Fig. 7e). These results revealed that most of genes in cyanobacteria could be inactivated by the pCyCBE system.

Discussion

Cyanobacteria are important microorganisms used in basic research and industrial biotechnology. As the first cyanobacterium strain with a sequenced genome[55], *Synechocystis* sp. PCC 6803 has been widely used as a host or model organism in synthetic biology studies. On the other hand, filamentous cyanobacterium *Anabaena* sp. PCC 7120 can form heterocysts for nitrogen fixation, and serves as the prominent model cyanobacterium to study nitrogen fixation and cell division. In this study, the pCyCBE plasmid was engineered, which enabled efficient C → T base editing in *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120. The base editing plasmid contains replicon RSF1010, which has a broad host range and is known to replicate well in diverse prokaryotes, such as *Synechococcus*[33], *Pseudomonas*[56], *Salmonella*[57], *Streptomyces*[58], *Rhizobium*[59], and *Mycobacterium*[58]. Therefore, it is assumed that the versatility of this editing system can be extended to other cyanobacterial species and even other prokaryotes. In the traditional allelic-exchange-based genome editing method, target genes are knocked out by inserting an antibiotic selection cassette, which may have polar effects on downstream genes in the operon. Using base editing, only a single nucleotide could be mutated, and marker-less gene inactivation was achieved without causing any polar effect. In addition, single base mutation by traditional method requires complex cloning steps and the selection of antibiotics marker[60], while the base editor could achieve scarless and precise base editing only by assembling a spacer at the target site. This greatly simplifies the construction of the editing plasmid. Besides the promising efficiency and precision of the base editing system, clean edits were observed in most colonies at the first round of selection. This suggested that the fully mutated strains could be quickly obtained without extra segregation steps. Moreover, the base editor harbored a defective Cas9 protein as the CRISPR effector to recognize the target site and did not generate DSBs in the cyanobacterial genome. Therefore, it exhibited low toxicity compared to CRISPR-Cas9 systems.

Recently, Wang et al. developed a base-editing system which employs dCas9 and the activation-induced cytidine deaminase (AID)[61]. This gene-editing tool could achieve efficient "C" to "T" base conversion at the positions 2 to 5 of the target site in the model cyanobacterium *Synechococcus elongatus* PCC 7942. In addition, a dCas12a-mediated base editing was also developed in *S. elongatus* PCC 7942 with a broader editable window (from positions 4 to 16 in the target spacer)[62]. In this study, we engineered the pCyCBE base editor plasmid by fusion of the cytidine deaminase APOBEC1 with the nickase Cas9D10A. This base editor enables efficient C → T conversion in *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120, and could achieve three-site editing simultaneously. These studies altogether demonstrate that base-editing is another powerful genetic tool for cyanobacteria.

Given that many cyanobacteria are polyploids and have a long generation time, serial genome editing of multiple genes in cyanobacteria is time-consuming. Multiplexed, simultaneous editing could drastically accelerate the genome-editing process. The pCyCBE system enables efficient base editing in multiplex sites simultaneously by simply assembling the tandem sgRNA cassettes. Thus, the pCyCBE system shows great potential to accelerate the metabolic engineering and synthetic biology research in cyanobacteria. Combined with massive parallel oligomer synthesis and high-throughput sequencing, the development of the pCyCBE system allows accurate genome-wide or defined gene library screening, which has been previously applied in human cells, *Corynebacterium glutamicum*, and *Bacillus subtilis*[63,64].

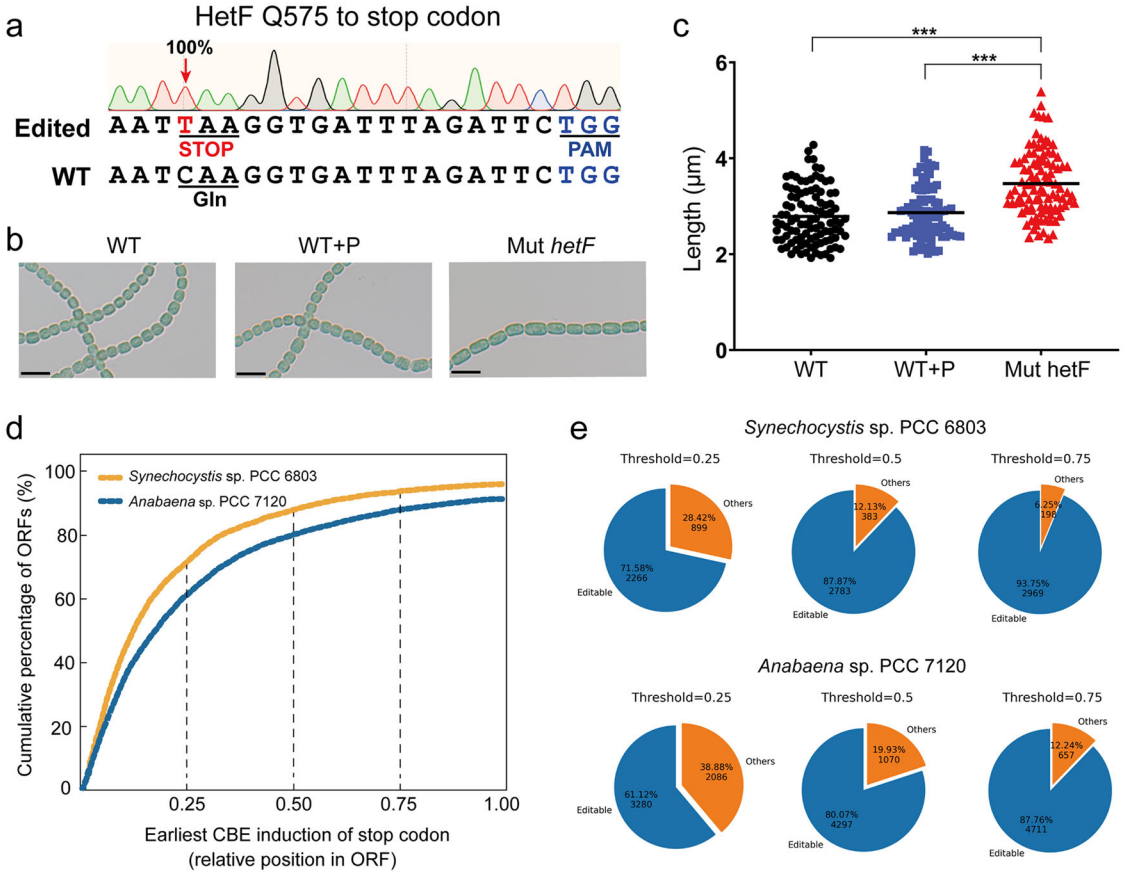

Fig. 7 | The pCyCBE base editing system can inactivate most genes in the cyanobacteria. a HetF Q575 in the genome of *Anabaena* sp. PCC 7120 is successfully mutated to stop codon. **b** Photographs of *Anabaena* sp. PCC 7120 wild-type (WT) strain, wild-type strain carrying the pCyCBE plasmid (WT + P), and the *hetF* mutant strain (Mut *hetF*). Bars = 10 μm. **c** The cell lengths of different *Anabaena* sp. PCC 7120 strains. 100 cells from each strain are measured under microscope. ***$p < 0.001$ is determined using Student's t test. **d** Relative position of the earliest induction of stop codons could be targeted in the ORFs of cyanobacteria (cumulative percentage) by the pCyCBE system. **e** The portion of the genes targeted by pCyCBE to introduce at least one premature stop codon within the top 25%, 50%, and 75% of the ORFs in *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120.

In summary, an efficient and convenient base editing system of pCyCBE plasmid is developed in cyanobacteria, which enables rapid C → T mutations at the specific genomic sites and introduces premature stop codon for gene inactivation. The pCyCBE system also enables simultaneous mutations at multiple genomic sites and can be cured easily by sucrose counter-selection. The pCyCBE system will not only expand the genome editing toolbox of cyanobacteria, but may also be helpful in the metabolic engineering and fundamental researches in cyanobacteria. The developed plasmid and the editing strategies presented in this study could be readily applied in other microorganisms and to other CRISPR systems.

Methods

Strains and culture conditions

All the strains used in this study are listed in Supplementary Table 3. *Escherichia coli* DH5α was used for molecular cloning, while *E. coli* DH10B carrying pRL443 and pRL623 was used for conjugation. All *E. coli* strains were cultivated in LB medium and on LB agar plates at 37 °C. The sub-strain of *Synechocystis* sp. PCC 6803 used in this study is a mobile and glucose-tolerant strain which is initially obtained from Professor Jindong Zhao's lab at Peking University. *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 strains were grown in BG11 liquid at 30°C under the light density of 30 μmol photons m^{-2} s^{-1}. The final Fe concentrations were 21.4 μM and 4 nM for the standard Fe and Fe-deficient conditions, respectively. BG11 plates were prepared by adding 0.3% $Na_2S_2O_3$, 1.4% agar, and 8 mM TES (pH 8.2) to the BG11 liquid medium. Antibiotics were used as follows: 50 μg/mL kanamycin, 50 μg/mL chloramphenicol, and 100 μg/mL ampicillin for the *E. coli* strains; 30 μg/mL kanamycin for the *Synechocystis* sp. PCC 6803 strains; and 50 μg/mL neomycin for the *Anabaena* sp. PCC 7120 strains.

Construction of editing plasmids

All the primers used in this study are listed in Supplementary Data 1. To construct the pCyCBE plasmid, components of cytosine base editor were amplified using the pBECKP-Km plasmid, and the RSF1010 origin was amplified using the pCpf1b-Sp plasmid (Supplementary Fig. 6a). Then the two amplified DNA fragments were assembled via Gibson assembly (Supplementary Fig. 6b). The successful construction of the pCyCBE plasmid was verified by PCR and Sanger sequencing.

To assemble the target spacer, a 20 bp-spacer sequence before NGG in the target gene of cyanobacteria was selected. The spacers were prepared by annealing a pair of complementary oligonucleotides, and ligating them into the *Bsa*I sites of the pCyCBE plasmid by Golden Gate assembly. Successful construction of plasmids was confirmed by PCR and Sanger sequencing (Supplementary Fig. 6c).

For the construction of two-site base editing plasmid, the DNA fragment of sgRNA1 was amplified from the constructed pCyCBE-sgRNA1 plasmid with the primers CBE2spF/CBE2spR (Supplementary Data 1). The pCyCBE-sgRNA2 plasmid was digested with the enzymes *Xba*I/*Not*I, and the digested product was purified using SanPrep Column PCR Product Purification Kit (Sangon, Shanghai, China). Then the DNA fragment of

sgRNA1 and the digested pCyCBE-sgRNA2 plasmid were ligated together via Golden Gate assembly. The constructed pCyCBE-2xsgRNA plasmid was verified by PCR and Sanger sequencing.

The construction procedure of three-site base editing plasmid was similar with that of two-site base editing plasmid. Briefly, the DNA fragments of sgRNA1 and sgRNA2 were amplified from the constructed pCyCBE-sgRNA1 and pCyCBE-sgRNA2 plasmids with the primers CBE3spF1/CBE3spR1 and CBE3spF2/CBE3spR2, respectively (Supplementary Data 1). Then the two DNA fragments were assembled into the *Xba*I/*Not*I sites of the pCyCBE-sgRNA3 plasmid via Golden Gate assembly.

Base editing in cyanobacterial strains

The constructed plasmids were transformed into *Synechocystis* sp. PCC 6803 by conjugation, as previously described[33]. The editing plasmid was first transformed into the DH10B strain carrying pRL443 and pRL623. Then the DH10B strain was cultured in LB broth containing 50 μg/mL kanamycin, 50 μg/mL chloramphenicol, and 100 μg/mL ampicillin, and was grown to $OD_{600} \approx 0.6$. 5 mL culture was harvested by centrifugation at 5000 rpm for 5 min, and was resuspended with 200 μL fresh LB medium after washing with fresh LB medium three times. 50 mL culture of wild-type *Synechocystis* sp. PCC 6803 ($OD_{730} = 1.0$~1.5) was harvested by centrifugation, and was resuspended with 2 mL BG11 medium after washing with BG11 medium three times. Then 100 μL of bacterial cells were mixed with 200 μL of *Synechocystis* sp. PCC 6803 strain, and the mixture was diluted 10^4 folds with fresh LB medium. 200 μL diluted mixture was poured onto the HAF Millipore filters (82 mm) overlayed on the BG11 agar plate. After 24 h incubation at 30°C under the light density of 20 μmol photons m^{-2} s^{-1}, the conjugation filters were transferred onto BG11 agar plate supplemented with 30 μg/mL kanamycin. Approximately 1000 colonies could be observed on the plate about one week later. The conjugation process of *Anabaena* sp. PCC 7120 strains was the same as that of *Synechocystis* sp. PCC 6803, except that the *Anabaena* cells were selected on the BG11 agar plate containing 50 μg/mL neomycin. Approximately 100 colonies of *Anabaena* sp. PCC 7120 could be observed on the plate one week later.

To assess the editing efficiency of the pCyCBE system, the colonies grown on the BG11 plate were collected as PCR template. PCR products covering the editing sites were sent to Shanghai Sangon for Sanger sequencing, and the editing efficiency was determined using EditR software[48]. The EditR software analyzes the fluorescence area of all four bases in the Sanger sequencing chromatogram to delineate the composition and frequency of base mutations.

Deep sequencing

The genomic DNA of mutant strains was extracted using a FastPure Bacteria DNA Isolation Mini Kit (Vazyme). About 400 bp regions surrounding the target loci were amplified using primers with barcodes. The PCR products were merged and gel-purified. The amplicon-seq library was prepared by using a TruSeq DNA Sample Prep Kit (Illumina). The library was subjected to Illumina MiSeq sequencing by Shanghai Majorbio Bio-pharm Technology Co. Ltd. The sequencing data were demultiplexed according to the barcodes, and the mutation efficiencies were analyzed using Cas-Analyzer[65]. The primers used in this study are listed in Supplementary Data 1.

Growth curve assay

For the Fe-deplete assay, glassware, tips, and bottles were all soaked in 6 M HCl for at least 12 h and subsequently rinsed with Milli-Q water six times before use. The *Synechocystis* sp. PCC 6803 wild-type (WT) strain and Mut *hlyD* strain were grown to logarithmic growth phase in BG11 media, and harvested by centrifugation, followed by washing with Fe-deplete BG11 medium (twice) to remove extracellular Fe. The WT and Mut *hlyD* strains were diluted using BG11 medium and Fe-deplete BG11 medium, respectively, with the final OD_{730} of 0.02. The growth curves of the cyanobacterial strains were monitored by measuring turbidity (OD_{730}) of three independent biological replicates every 2 days until the 12th day. The photographs of the strains were taken on the 6th day.

Phototaxis assay

The phototaxis assay was performed by following the method described previously[46]. Briefly, *Synechocystis* sp. PCC 6803 WT and Mut *hlyD* strains were both grown on solid BG11 plates containing 1.5% (w/v) agar, and then streaked onto solid BG11 plates containing 0.8% (w/v) agar with a sterile toothpick. The plates were then wrapped in black cardboard and incubated at 30°C with only one side exposed to unilateral light (5 μmol photons m^{-2} s^{-1}). After 7 days of incubation, the movement of *Synechocystis* strains on the plates was observed and photographs were taken.

Plasmid curing

To cure the pCyCBE plasmid after successful editing, the strain to be cured was grown in antibiotic-free BG11 liquid medium till 4th generation. The cell culture ($OD_{730} = 1.0 \sim 1.5$) was then serially diluted and spread onto the sucrose-free BG11 plate and the BG11 plate containing 5% w/v sucrose, simultaneously. To check the loss of the editing plasmid, colonies formed on the sucrose plate were randomly picked, and streaked on the antibiotics-containing plates. Loss of the editing plasmid was further confirmed by PCR.

Statistics and reproducibility

The statistical analyses conducted on the data in each figure were described in their respective figure captions. All analyses were conducted with the software package GraphPad Prism 8.2.1 (GraphPad, San Diego, CA). Experiments were performed with three independent repeats.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data are available in the main text or the supplementary materials. The raw data of deep sequencing were deposited in the NCBI Sequence Read Archive (SRA) with the accession number SAMN41576882. The source data underlying Figs. 3, 4, 7, and Supplementary Fig. 3 can be found in Supplementary Data 2.

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Author contributions

H.B.J. and W.C. conceived and designed the experiments. X.D.L., L.M.L., Y.C.X., Q.W.S., Z.L., H.L.H, X.W.W, and W.C. performed the experiments. X.D.L. and W.C. analyzed the data. X.D.L., H.B.J., and W.C. wrote the manuscript. H.B.J. and W.C. supervised the research project. All authors contributed to the article and approved the submitted version.

Competing interests

The authors declare no competing interests.

Additional information

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